

Inhibition of Monocarboxylate Transporter-1-Mediated
Lactate Uptake by Tea Catechins

A Senior Honors Thesis

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by

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Introduction

Tea and Cancer

Tea is one of the most consumed beverages on Earth, second only to water. In 2010, over 3 billion gallons of tea were consumed by Americans, with black tea and black tea accounting for 80% and 19.5%, respectively (1). Tea consumption has been associated with a positive effect on certain diseases, including arthritis, HIV, and cancers of the gastrointestinal (GI) tract (2,3). In many populations, including Japanese women, the risk of gastric cancer decreases with increased consumption of tea (4). Green tea has also been shown to reduce inflammation of the gastric epithelium, which is a significant risk factor for gastric cancer(5). Of the many compounds found in green tea, the catechins, a group of polyphenols, are considered to be the primary bioactive components (Fig. 1).

There have been many studies on the effects of catechins on the human body, especially epigallocatechin gallate (EGCG), one of the abundant catechins in green tea. EGCG has many effects in the GI tract, such as inhibiting NF- κ B activity (6). NF- κ B is a protein that is responsible for many inflammatory pathways, and thus the inhibitory effects of tea catechins may be the basis by which gastric cancers are prevented by green tea. Green tea has also been associated with the inhibition of cancer cell proliferation in the stomach, which is often attributed to its antioxidant activity (7). Whether this is the sole pathway is controversial. Furthermore, the mechanism by which EGCG and other catechins are taken up by the stomach tissue is still unclear.

Cancer Proliferation

For a tumor to grow, its cells need to undergo mitosis, and as such they require vast amounts of energy and nutrients. Tumors are made up of cells that often differentiate due to their access to the blood stream and essential nutrients. These differentiated cell types generally integrate their metabolism of nutrients (Fig. 2). Cells that are distal to the vasculature (hypoxic cells) will metabolize glucose and efflux lactate. This lactate is taken up by oxidative cancer cells by the monocarboxylate transporter-1 (MCT-1) with preferential metabolism compared to glucose (8). Some studies have shown that this metabolic relationship can be disrupted by inhibiting MCT-1, which would therefore reduce the proliferation of these tumors (9).

Monocarboxylate Transporters

Monocarboxylate transporters (MCT) are a family of proteins that are found in the plasma membrane of many cell types. At least 5 MCT isoforms have been isolated, with MCT-1 being the first discovered and also the most thoroughly characterized. MCT-1 transports small monocarboxylates such as lactate, pyruvate, and butyrate into the cell by a proton-dependent mechanism. (10) While present on the apical surface of much of the GI tract, it is only expressed on the basolateral membrane of the gastric epithelium (11). Of the remaining MCTs, MCT-2 and MCT-4 have been the most studied. MCT-2 has a similar function to MCT-1, but has a higher affinity for pyruvate and lactate (10). MCT-2 expression is low in normal tissue, but can be elevated in cancer tissue (10). Like MCT-1, MCT-4 is responsible for the efflux of monocarboxylates from the cell. Some, but not all, studies have suggested that MCT may play a role in catechin uptake into the small intestine (12, 13). MCT is also being studied as a potential method to disrupt the metabolism of tumor cells (14).

BCECF Assay

Developed by Halestrap, the BCECF assay uses the intracellular, pH-dependent, fluorescent dye 2',7' -bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) to indirectly measure lactate uptake into the cell. Cells are exposed to the acetylmethylated ester BCECF (BCECF-AM). The hydrophobic ester diffuses across the cell membrane and is de-esterified in the cytosol by endogenous esterase activity. Once de-esterified, the anionic form undergoes structural changes based on the internal pH of the cell, with each form fluorescing at different intensities. Cells are then exposed to lactate and pH is determined by fluorimetry. One can also treat the cells with inhibitors of MCT to assess inhibition of lactate uptake. This assay has primarily been done with suspended cell types such as Ehrliche-Ascites tumor cells, whereas data with adherent cell types is minimal (15).

Phytochemicals and the Stomach

The stomach, compared to the intestines and even the oral cavity, is rarely thought of as an organ that absorbs nutrients. This is a rather narrow-minded view, as it is known that alcohol and other drugs are absorbed through the stomach lining (16, 17). In fact, recent evidence from mouse models has shown that a group of phytochemicals known as anthocyanins can be absorbed through the stomach (18). This raises the question of what other nutrients and xenobiotics may be absorbed in the stomach.

The Present Study

The objective of my thesis project was to determine the effect of catechins on lactate uptake in two human gastric cells lines, i.e., the gastric adenocarcinoma cell line AGS and a

gastric carcinoma cell line derived from a metastasis in the liver (NCI-N87) (Fig. 3). The NCI-N87 cell line is unique in that it develops into a highly differentiated monolayer that displays an exemplary epithelial phenotype, including characteristics such as mucin production and expression of enzymes such as gastric lipase (19). In order to determine a pathway through which cancer proliferation is inhibited by tea, catechins have been used to determine their inhibitory effects on lactate uptake compared to phloretin, a known inhibitor of MCT. This will be done in a variation of Halestrap's BCECF assay, which has been adapted for use with adherent cell lines.

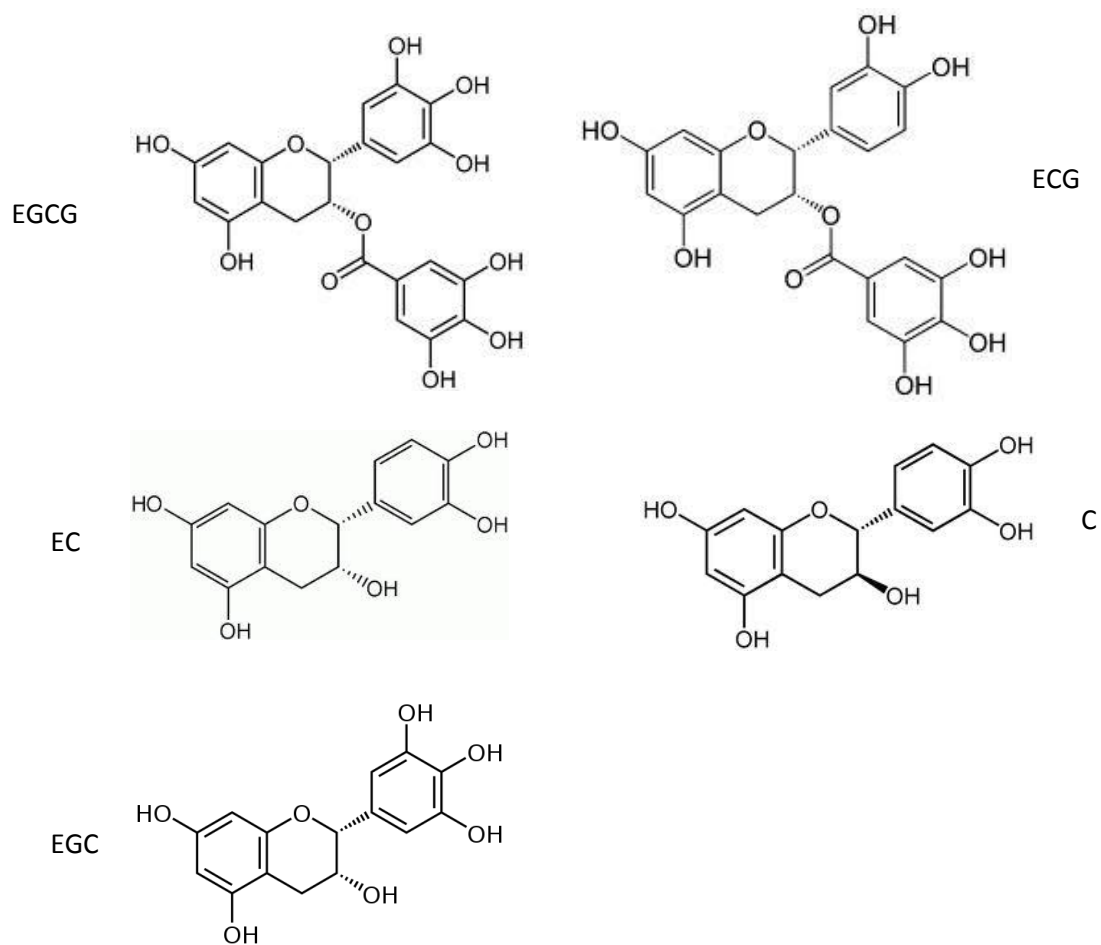


Figure 1: The Catechins.

Catechin (C), Epicatechin (EC), Epigallocatechin (EGC), Epicatechin gallate (ECG), and Epigallocatechin gallate (EGCG).

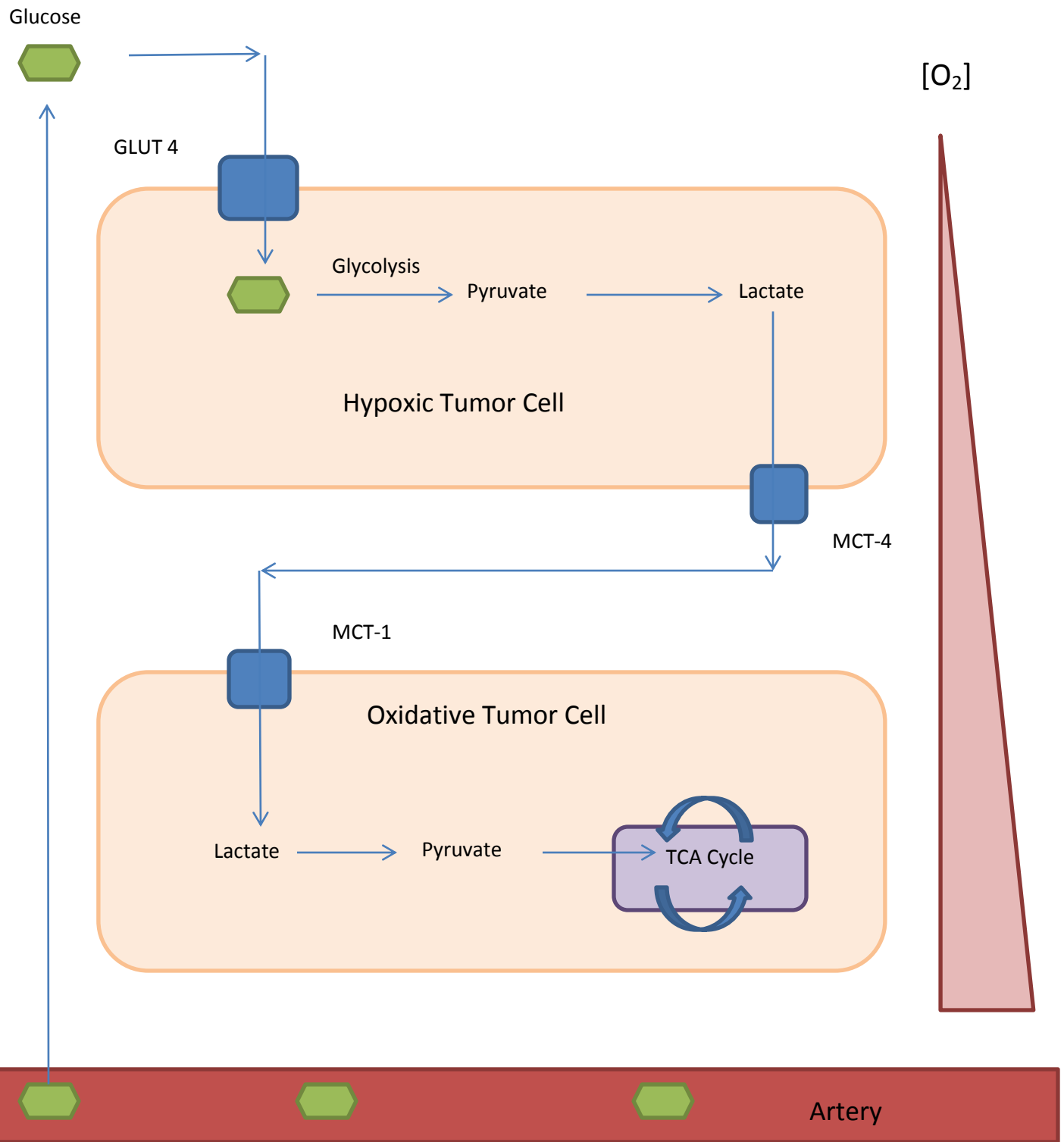


Figure 2: Tumor Metabolism. In tumors, cells express hypoxic and oxidative phenotypes, that work together to metabolize glucose and its products. This relationship is largely dependent on MCTs. Based on Semenza *et al* (2008).

Materials and Methods

I- Materials

BCECF-AM was purchased from Invitrogen. EGCG, phloretin, albumin, and lactate were purchased from Sigma Aldrich. All cell culture equipment was purchased from Fisher Scientific. All experiments were performed in Falcon 48-well plates.

Assay buffer for the BCECF assay was prepared as previously described by Halestrap *et al* (15). Final concentrations were as follow: 150 mM Na⁺, 5 mM K⁺, 150 mM Cl⁻, 1 mM Ca²⁺, 1 mM sulfate, 1 mM phosphate, 3.3 mM Mops, 3.3 mM Tris, and 5 mM HEPES, adjusted to pH 7.4 at 25 °C.

II- Cell Culture

AGS cells and NCI-N87 cells were purchased from ATCC. AGS cells were grown in DMEM with 10% FBS, 1% Penicillin/Streptomycin, and 2 mM L-glutamine. NCI-N87 cells were grown in RPMI 1640 with 10% FBS and 1% Penicillin/Streptomycin). All cultures were maintained in an atmosphere of 95% air: 5% CO₂ at 37°C. Cultures were split when cells were 90 % confluent by exposing the cells to 0.25% trypsin-EDTA. Cell concentration was determined with a hemocytometer. 48-well plates were seeded at a density of 2.0×10^5 cells per mL. Passages 5-21 were used for the subsequent studies. AGS wells were used once confluency was reached. NCI-N87 wells were used 18 days-post confluency.

III- BCECF concentration titration

In order to determine the appropriate concentration of BCECF, cells were exposed to various concentrations of BCECF and then read in a fluorometer. Confluent cultures

were treated with 4 μ M, 10 μ M, and 40 μ M BCECF or blank without media for 30 minutes at room temperature. Fluorescence was determined using an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

IV- MTT Assays for phloretin

To examine the cytotoxic effects of these phytochemicals, an MTT assay was performed. Briefly, media was removed from confluent cultures and the cells were incubated in medium containing phloretin (0 μ M, 3.13 μ M, 6.25 μ M, 12.5 μ M, 25 μ M, 50 μ M, and 100 μ M) and incubated at 37°. After 48 hours, medium was removed and the cells were incubated with medium containing yellow tetrazolium MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 15 μ L, 5 mg/mL) for 3 hours. The MTT is reduced by mitochondrial dehydrogenase enzymes, resulting in a purple precipitate. Medium was aspirated and the monolayer was washed with phosphate-buffered saline. This precipitate was dissolved in 150 μ L 0.04N HCl-isopropanol, and the absorbance was read at 610 nm.

V- Lactate Titration

Three concentrations of lactate were tested in order to determine the concentration needed for appropriate intensity of the signal in the BCECF assay. Confluent cultures were incubated at 6° C for 20 minutes before spent medium was removed. 500 μ L BCECF (10 μ M) in assay buffer was added to each well, and cultures were incubated at room temperature for 30 minutes. Buffer was removed and the cells were then washed once with assay buffer, twice with albumin (2 g/L) in assay buffer, and then again with

assay buffer. The cells were then exposed with 500 μ L of lactate (2 mM, 10 mM, 25 mM) in assay buffer. Dishes were immediately placed in a fluorometer and analyzed at an excitation wavelength of 530 nm and an emission wavelength of 590 nm for 20 minutes.

VI- Lactate Uptake and Inhibition by EGCG

In order to measure lactate uptake and its inhibition by EGCG, a modified version of the BCECF Assay described by Halestrap was used. Phloretin, a known inhibitor of MCT, was used as a positive control. AGS cells were then incubated at 6°C for 20 minutes. After incubation, spent media was withdrawn and the cells were treated with 500 μ L BCECF (10 μ M) in assay buffer, except for BCECF- control wells, which received assay buffer only for the rest of the procedure. The treated cells were left at room temperature for 30 minutes. The cells were then washed once with assay buffer, twice with albumin (2 g/L), and then again with assay buffer. Following the wash, control wells were pretreated with 500 μ L phloretin (200 μ M) in assay buffer, EGCG (200 μ M) in assay buffer, or assay buffer for 2 minutes. The wells were then placed on ice and incubated with 500 μ L assay buffer. Treatment wells were pretreated with 500 μ L phloretin (200 μ M) in assay buffer, EGCG (200 μ M) in assay buffer, or assay buffer for 2 minutes after the wash, and then exposed with 500 μ L lactate (10 mM) in assay buffer. Immediately following treatment with lactate or assay buffer, the fluorescence was measured at an excitation of 530 nm and emission of 590 nm for 15 minutes.

The process above was identical for NCI-N87.

VII- BCECF Assay with Various Catechins

In order to test the effect of other tea catechins on lactate uptake, the BCECF assay was repeated using EGCG (200 μ M), epigallocatechin (EGC, 200 μ M), epicatechin gallate (ECG, 200 μ M), epicatechin (EC, 200 μ M), and catechin (C, 200 μ M). The cells were treated and fluorescence was measured as above.

VIII- Statistics

Statistical significance was calculated using Student's t-test. Results with $p < 0.05$ were deemed statistically significant. For BCECF and lactate concentration experiments, $n=4$. For MTT and BCECF assays, $n=6$.

Results

I- BCECF Concentration

With increasing concentrations of BCECF in the assay buffer, cellular fluorescence increased. The ratio of change in concentration over change in fluorescence for 4 μM to 10 μM was 23 fluorescence units/ μM compared to 12 fluorescence units/ μM for 10 μM to 40 μM . (Fig. 4) Thus, 10 μM was chosen as the concentration to be used in subsequent experiments.

II- MTT Assay for Phloretin

As concentration of phloretin increases, the percentage of absorbance, and therefore viable cells. Exposure of cells to as high as 25 μM phloretin did not significantly reduce respiratory activity. However, respiratory activity was significantly decreased following incubation in medium containing 50 μM and 100 μM phloretin, with declines of 18% and 52%, respectively (Fig. 5). Observation of cells after MTT assay revealed that cells were still viable up to 100 μM .

III- Lactate Titration

BCECF fluorescence, and thus intracellular pH, was altered during exposure to assay buffer containing 2-25 mM lactate. The response was dose-dependent with the most marked change observed during the initial 10 minutes of incubation. (Fig. 6)

IV- Lactate Uptake and Inhibition with EGCG

In the AGS cell line, exposure of cultures to assay buffer containing EGCG and phloretin significantly inhibited lactate uptake, as decrease in fluorescence was attenuated compared to

cultures incubated with lactate only (Fig 7). The response of EGCG-treated cells was slightly less than that in cultures treated with equivalent phloretin, but still far greater than that of lactate alone. The difference in extent of inhibition of lactate uptake was intensified in the NCI-N87 cells (Fig. 8).

V- BCECF Assay with Various Catechins

The response of cells to the presence of catechins was dependent on structure, with each catechin showed a varying degree of inhibitory effect. Catechin (C) had the greatest inhibitory effect on lactate uptake as fluorescence decreased by 16% compared to 31% with the negative control upon exposure to lactate (Fig. 9). EGCG also significantly decreased lactate uptake compared to the control. In contrast, EC, ECG, and EGC failed to significantly affect lactate uptake.

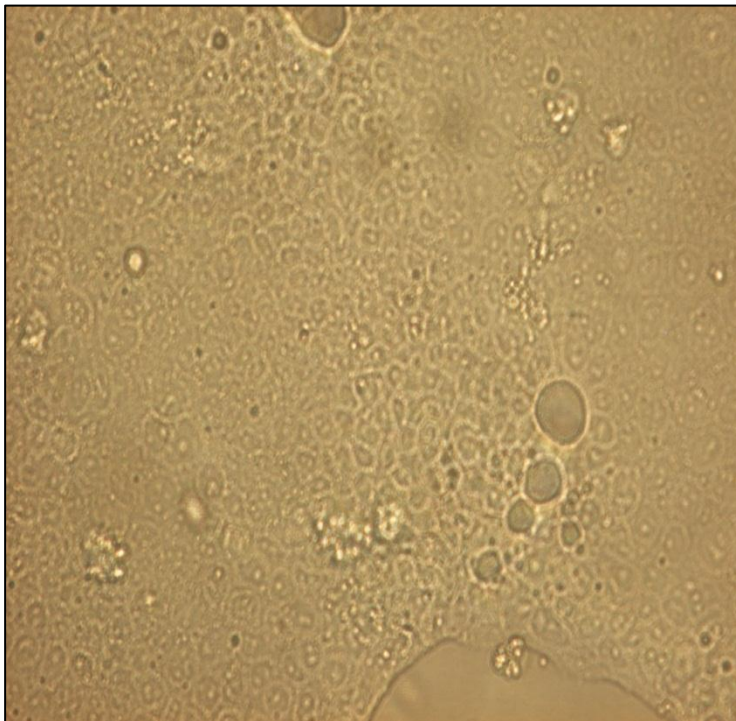
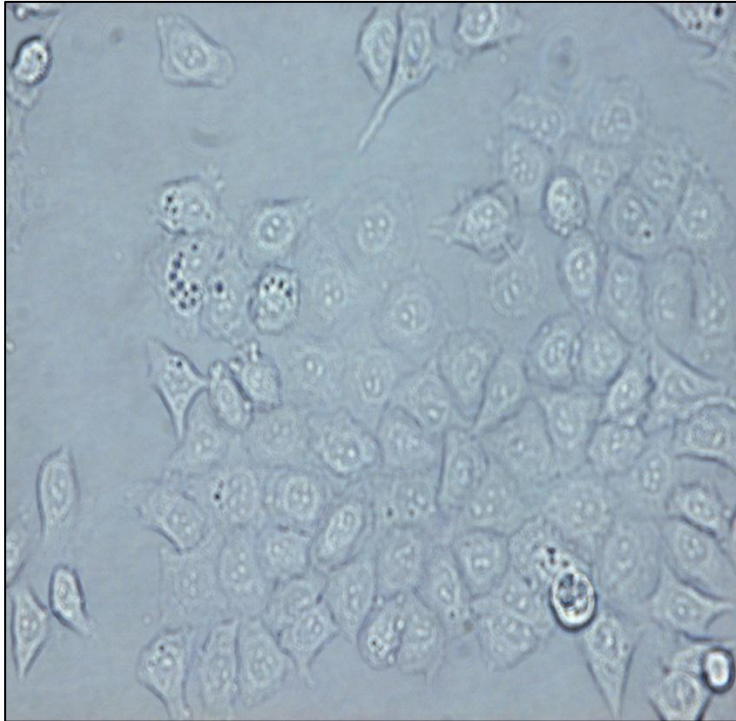


Figure 3. The cell lines used in this study were the AGS (top) and NCI-N87 (bottom).

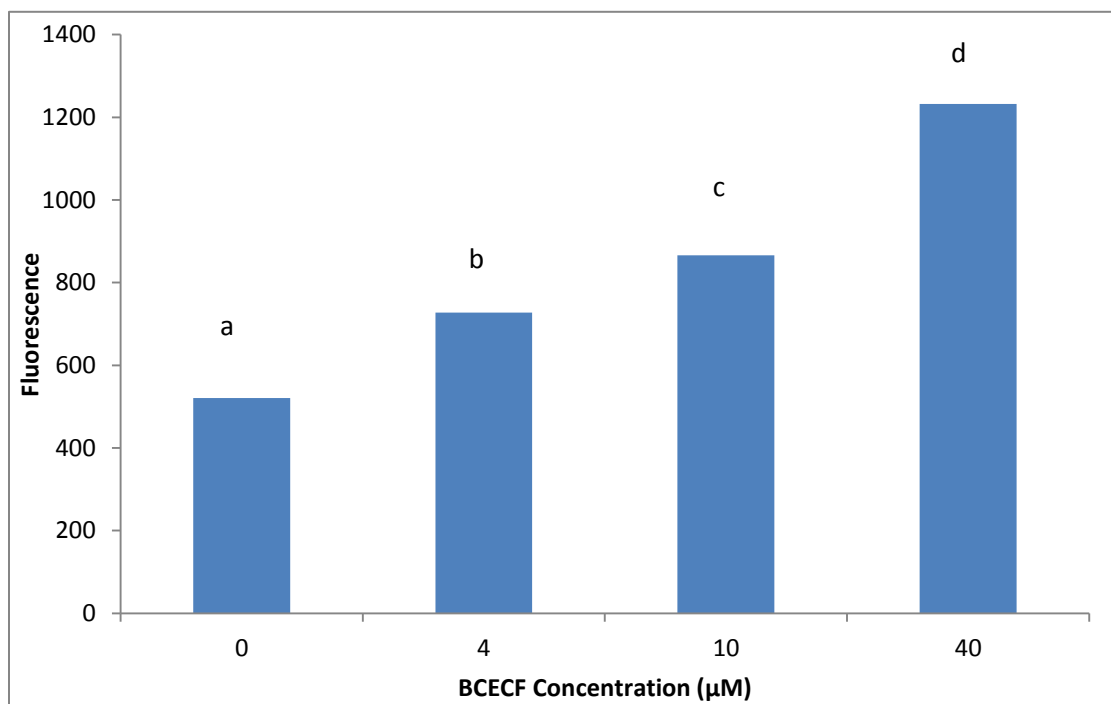


Figure 4: BCECF Titration in AGS cell line. Bars with different letters are considered statistically significant with a p-value <0.05.

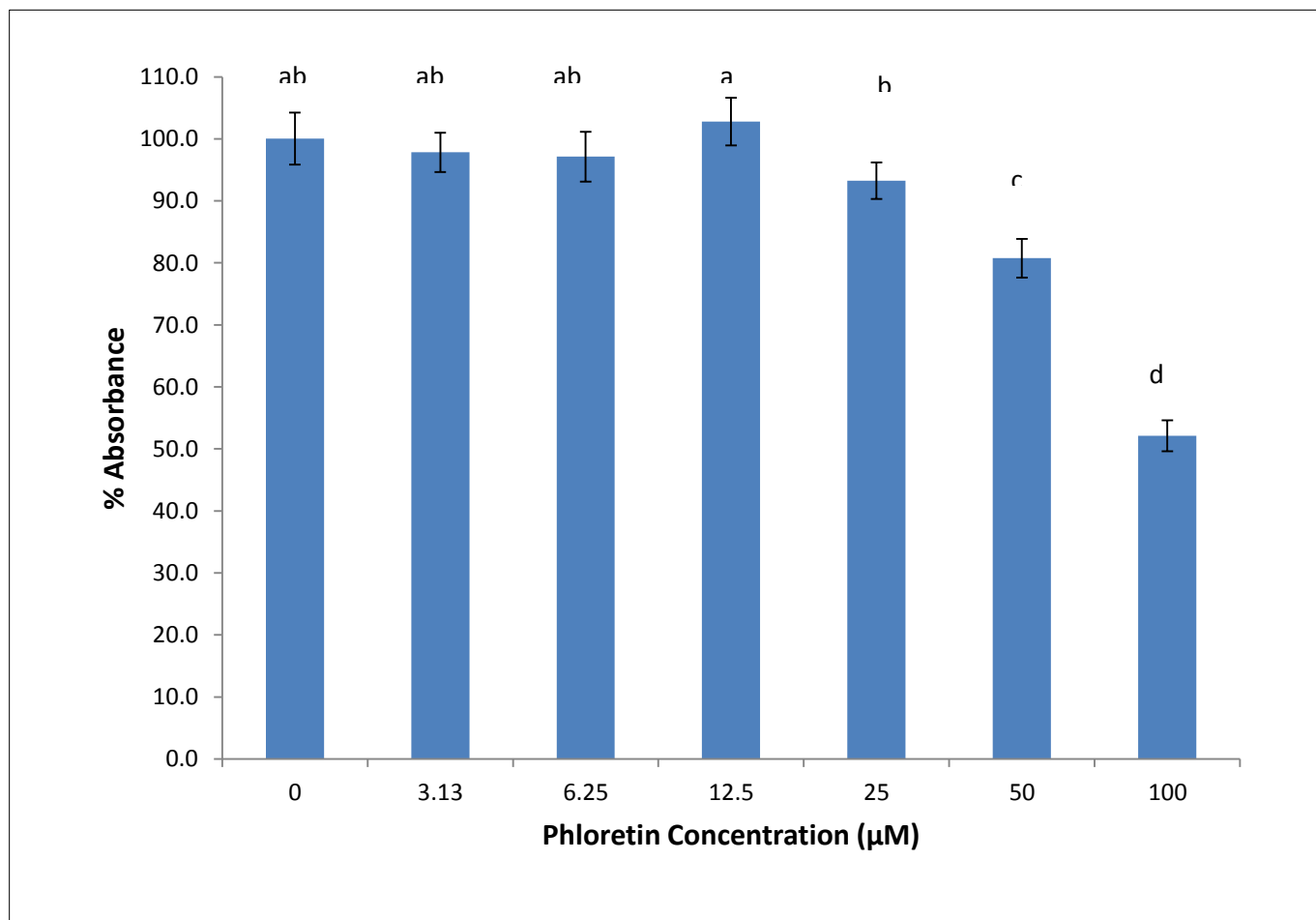


Figure 5: MTT Assay in AGS cell line. Bars with different letters are considered statistically significant with a p-value <0.05 .

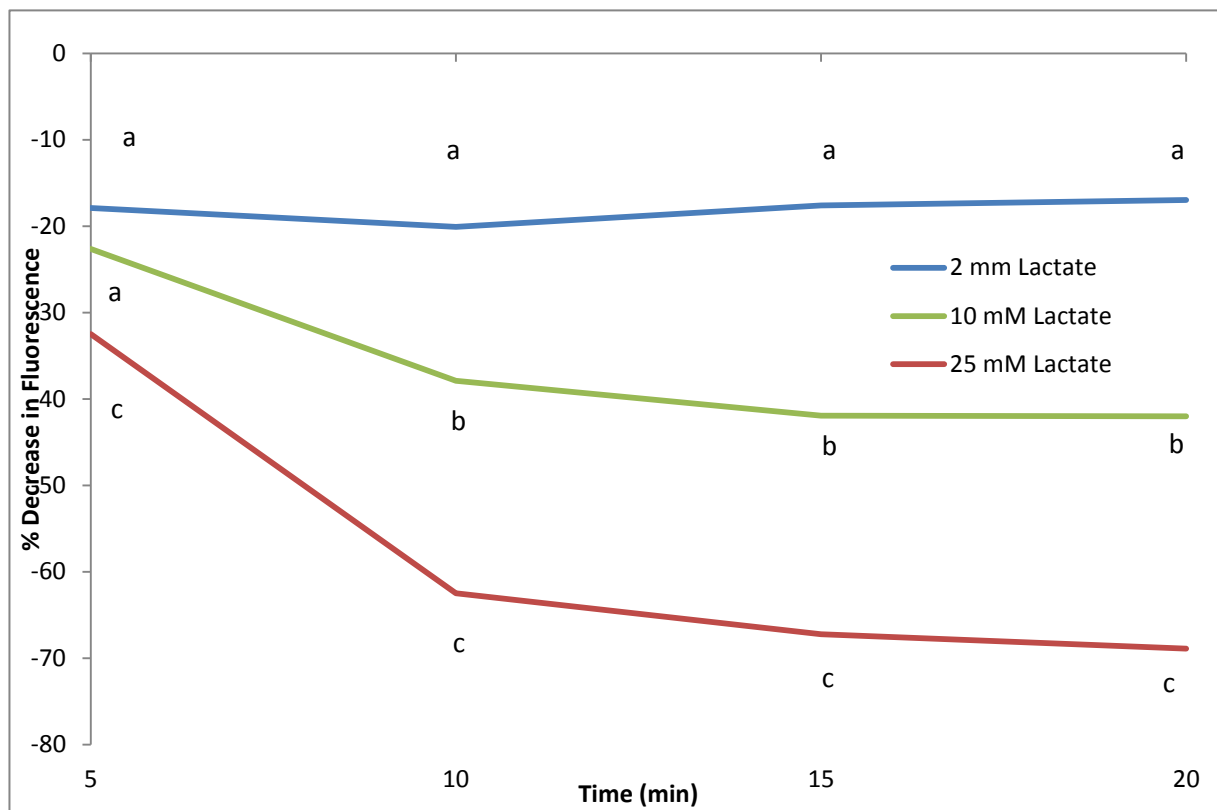


Figure 6: Lactate Titration in AGS cell line. Points with different letters are considered statistically significant with a p-value <0.05.

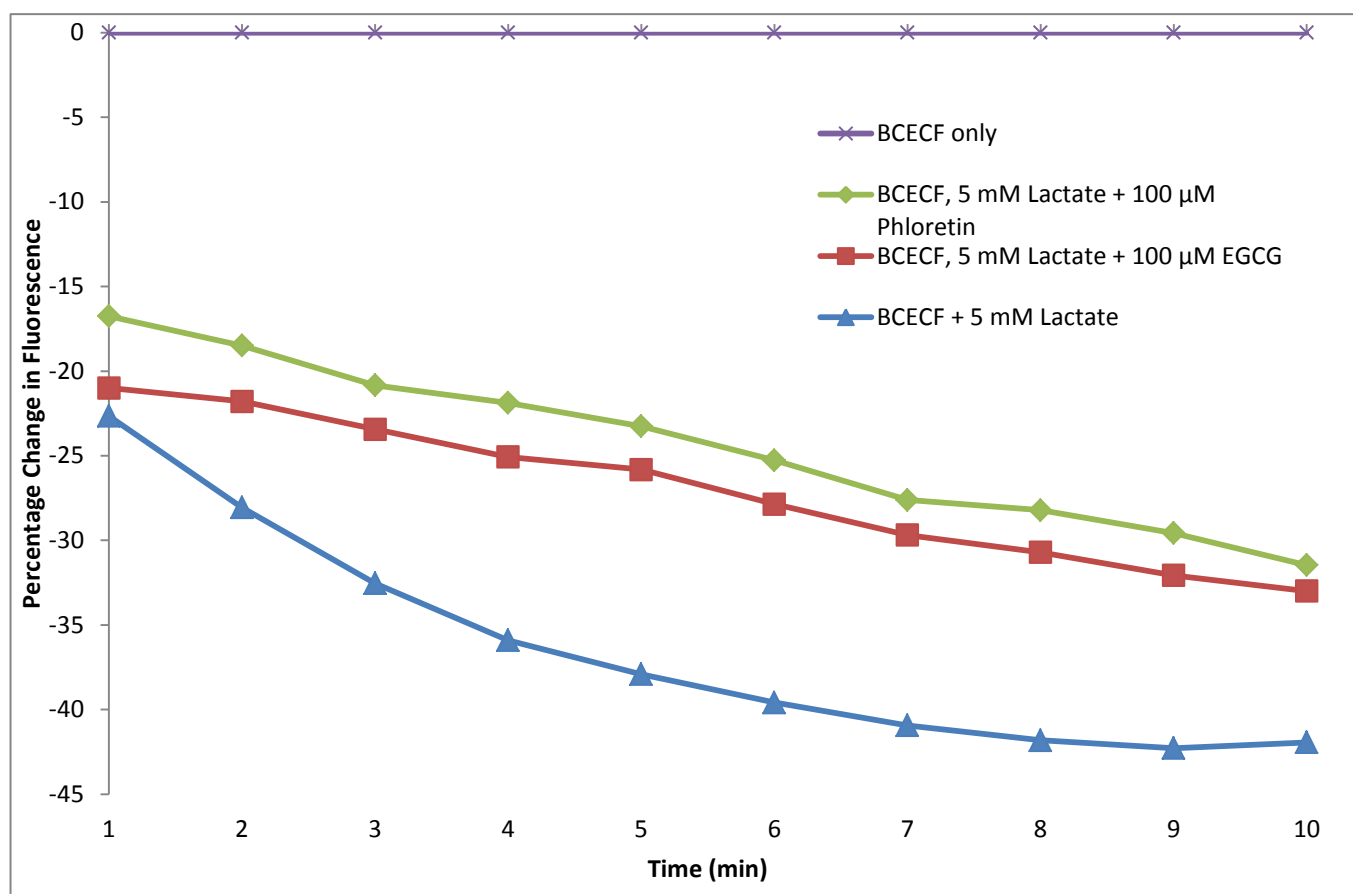


Figure 7: EGCG, like phloretin, inhibits the reduction in intracellular pH in AGS cells.

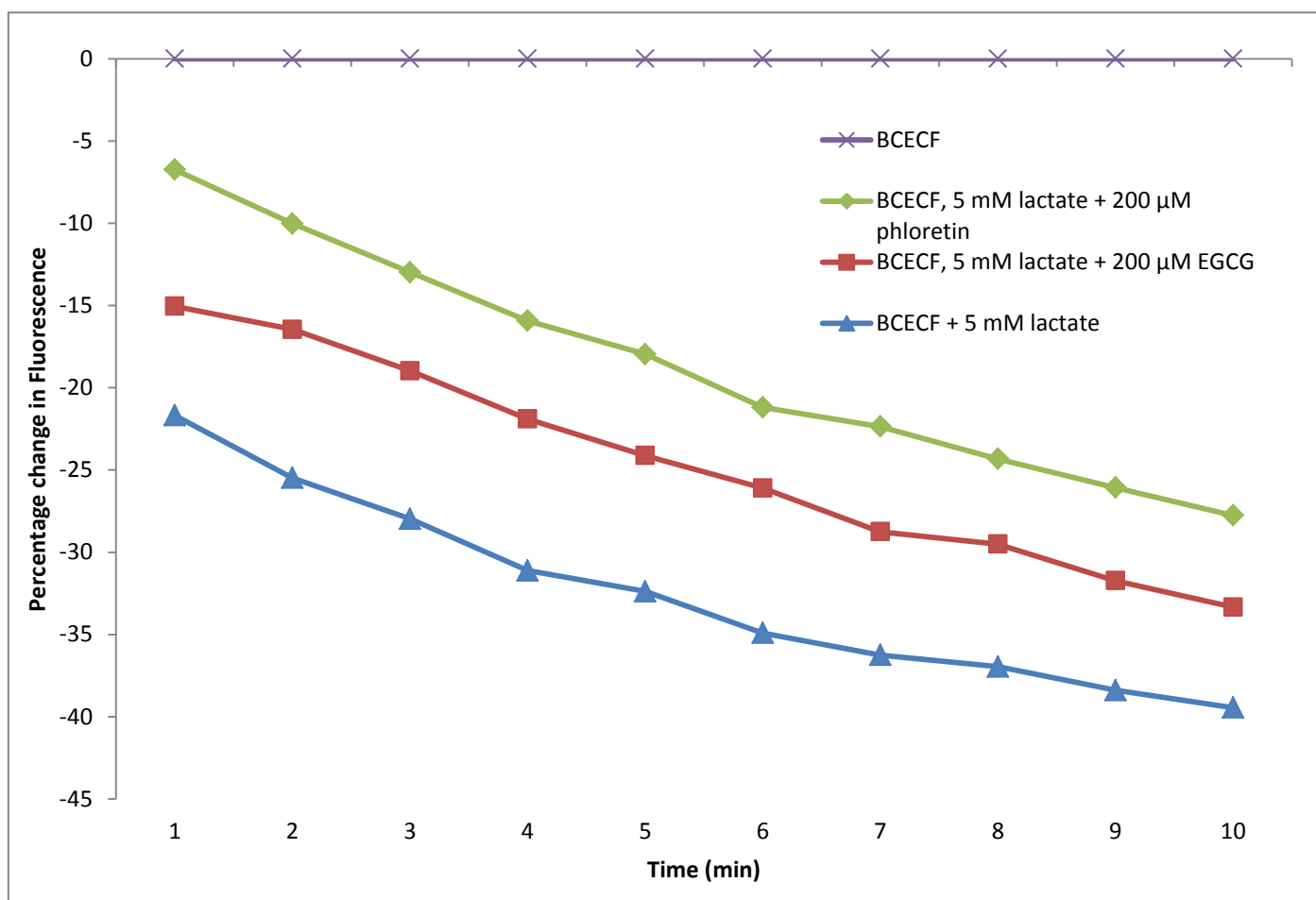


Figure 8: EGCG, like phloretin, inhibits the reduction in intracellular pH NCI-N87 cells.

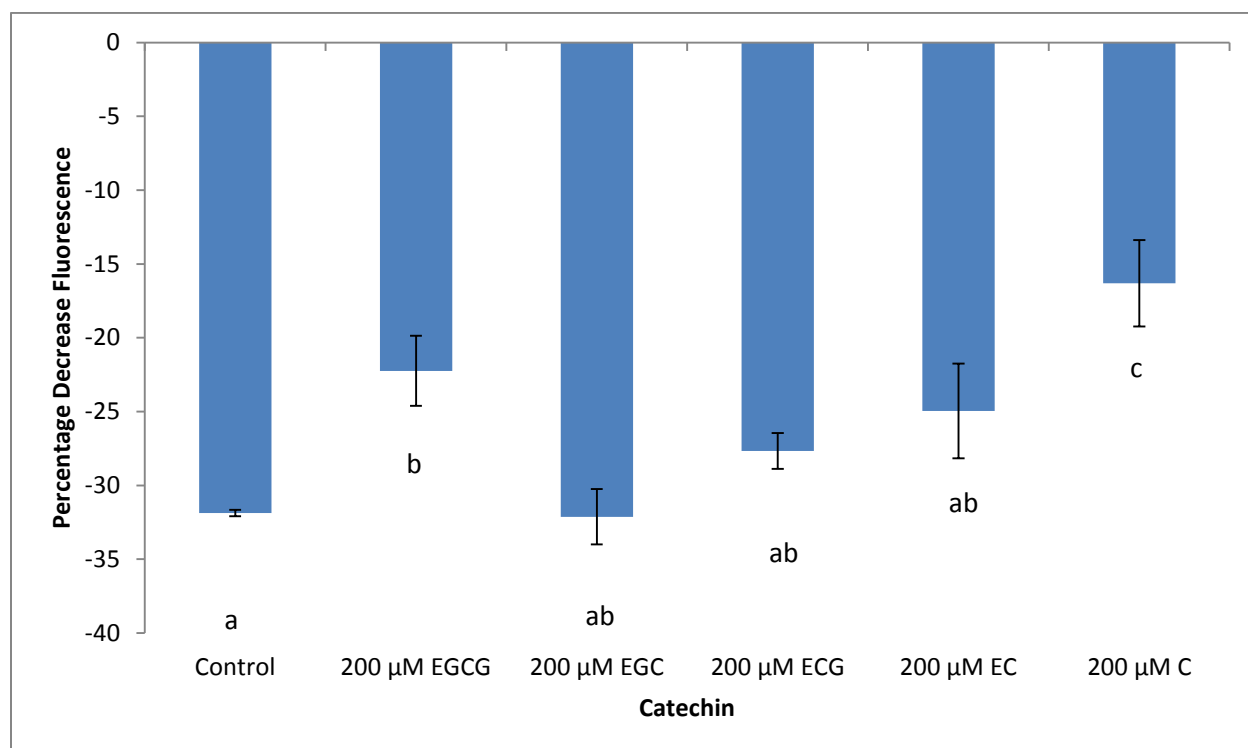


Figure 9: Differential effects of catechins on lactate uptake in AGS cells. Points with different letters are considered statistically significant with a p-value <0.05.

Discussion

The first step of this study was to adapt the BCECF assay developed by Halestrap for investigating lactate uptake to be used in adherent cell lines. The original protocol for this assay involved the cells being constantly read while continuously spinning in a cuvette. The cell lines used in this study were grown on 48-well dishes. In order to account for any background fluorescence, all readings were corrected by subtracting the fluorescence emitted by cells without BCECF. In fig. 4, various concentrations of BCECF were used to determine the appropriate amount for this assay. The concentration of 10 μM was chosen for the level of fluorescent signal, the minimal increase at 25 μM , and the cost of the dye. In order to assess the optimal concentration of phloretin that could be used as an inhibitor, an MTT assay was performed. Although a level of 200 μM was shown to be cytotoxic after 48 hours (fig 5.), this was well below the level used in previous Caco-2 models. In addition, as the cells were only exposed to phloretin for less than 20 minutes, this concentration was considered to be safe. Concentrations of catechins were chosen based on the concentration of phloretin. Lactate was also tested at various concentrations, with 25 mM being the initial suggested amount by Dr. Halestrap. More dilute solutions were tested, and the 10 mM solution provided a response that was useful over the 15 minute intervals, although it was not as robust as the 25 mM solution. This was considered so that a high concentration of lactate would not saturate MCT and offset the inhibitory effect of the catechins. Altogether, these changes to the BCECF assay allowed us to successfully measure lactate uptake in our adherent cell lines.

The data generated by the BCECF assay support our hypothesis that catechins have an inhibitory effect of lactate uptake. The fact that this effect is reproducible in two different cell types that are phenotypically distinct also supports this conclusion. Of the two cell types, NCI-

N87 provides the better model for this study, as it has a phenotype that is more characteristic of the stomach epithelium, such as forming tight junctions and producing gastric mucin. The inhibition of MCT as a mechanism for inhibiting tumor growth is being researched as a possible therapeutic method for treating cancer, and this model shows that tea compounds might have a similar, though much smaller, effect on gastric tumors.

Of the tested catechins, only catechin and EGCG produced a statistically significant inhibition of lactate uptake. Epicatechin and epicatechin gallate produced a slight, but insignificant, inhibition. Catechin and epicatechin are both much smaller than the other compounds, and they also share a strong structural similarity to phloretin. The structure of these three compounds, especially the 5,7, and 4' hydroxyl groups on the catechins that are parallel to the 4, 2', and 4' hydroxyl groups on phloretin. The inhibitory effects of EGCG and ECG are more surprising, as the addition of the large gallate group make these compounds much larger than the other catechins. However, the inhibitory effect of EGCG compared to that of EGC, which is only different in that it lacks the gallate group, suggests that the gallate group on these compounds might promote the inhibitory effect.

Two major concerns are raised when proposing inhibition of lactate uptake mediated by MCT-1 as a possible mechanism for the epidemiological data that show that in certain populations, gastric cancer rates are decreased with increased consumption of tea. First, many reports suggest that MCT-1 is only found on the basolateral surface of the stomach epithelium. While there are data supporting this claim, there is also evidence that MCT-1 can also be expressed on the apical membrane as well in cancer cells. Also, other phytochemicals, primarily anthocyanins, have been shown to be taken up into the gastric epithelium by other proteins such as bilitranslocase, and even transported across the basolateral membrane. If this is so for

catechins, these compounds could be located throughout the stomach tissue. The second issue is whether MCT-1 is actually being targeted by phloretin and the catechins and if it is the protein facilitating the lactate transfer, to decrease intracellular pH. Further studies are needed to support these data.

In subsequent studies, this assay will be performed using a synthetic inhibitor with a high specificity and inhibitory effect on MCT-1 to determine if MCT-1 is the protein responsible for this effect. Further studies are also being done to determine if MCT-1 is involved in the transport of catechins into the cell. This can be done with high performance liquid chromatography (HPLC) to determine if the amount of catechins and catechin metabolites inside the cell and in medium change upon addition of the synthetic inhibitor. This would ideally be done with the NCI-N87 line grown on inserts. In this method, the low pH of the stomach can be simulated along with the benefits of having a more physiologically relevant phenotype.

Monocarboxylate transporters are an exciting class of proteins that could become a novel method for the treatment of and prevention of certain kinds of cancers. Tea is one of the most commonly consumed beverages around the world, and its bioactive compounds, the catechins, have been shown epidemiologically to possess cancer-preventative properties in certain populations. These results suggest that catechins have an inhibitory effect on lactate uptake via MCTs, which may be one mechanism by which cancer is prevented and its growth inhibited in these populations.

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